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Molecular and biochemical diagnosis of esterase-mediated pyrethroid resistance in a Mexican strain of *Boophilus microplus* (Acari: Ixodidae)

FELIX D. GUERRERO*, JOHN H. PRUETT and ANDREW Y. LI
USDA-ARS, Knipling-Bushland US Livestock Insects Research Laboratory, 2700 Fredericksburg Rd., Kerrville, TX 78028, USA

Abstract. We examined pyrethroid resistant Mexican strains of *Boophilus microplus* using biochemical and molecular tests to determine the mechanisms conferring resistance. Permethrin hydrolysis assays and esterase activity gels indicated enhanced esterase-mediated metabolic detoxification in the Cz strain, while one other pyrethroid resistant strain, SF, and two pyrethroid susceptible strains had lower levels of permethrin hydrolysis. Results from assays using a PCR-based test to detect a pyrethroid target site resistance-associated mutation in the tick sodium channel gene found only low levels of mutations in the Cz strain, while the SF strain had a high level of the mutated sodium channel alleles. A specific esterase, designated CzEst9, believed to be responsible for the esterase-mediated pyrethroid resistance in the Cz strain was purified, and the gene encoding CzEst9 cloned.

Key words: pyrethroid resistance mechanism, sodium channel mutation, polymerase chain reaction, diagnostic assay, metabolic esterase

Introduction

The southern cattle tick, *Boophilus microplus* (Canestrini), was eradicated from the United States during a 55-year program coordinated by the United States Department of Agriculture (Graham and Hourrigan, 1977) and the US remains free of *B. microplus* through a USDA-APHIS/VS program established at the border of the United States and Mexico. Large numbers of cattle are imported annually into the United States from Mexico and the continued ability to control tick populations on the ranches of Mexico will be a major factor in the United States remaining *Boophilus*-free. Mexican cattle producers have made extensive use of pyrethroid-based pesticides in attempting to control *B. microplus* and problems with pesticide resistance have begun to intensify.

*Author for correspondence (E-mail: felixg@ktc.com);

There are several mechanisms which can lead to pyrethroid resistance in arthropods. The target of pyrethroids is the sodium channel and several mutations in arthropod sodium channel gene coding regions have been shown to cause pyrethroid resistance (Vais *et al*., 2000; Tan *et al*., 2002). In *B. microplus*, a nucleotide substitution in the sodium channel gene coding region leading to a Phe → Ile amino acid substitution in the S6 transmembrane segment of domain III has been associated with pyrethroid resistance (Guerrero *et al*., 2001). However, toxicological studies of several pyrethroid resistant Mexican strains of *B. microplus* indicated that, in addition to target site insensitivity, metabolic resistance to pyrethroids was also an important mechanism (Miller *et al*., 1999). Esterases are a family of enzymes, which can be involved in metabolic-based pesticide resistance through their ability to sequester or hydrolyze various substrates. Jamroz *et al*. (2000) examined general esterase activities in Mexican strains of *B. microplus* and reported a pyrethroid resistant strain, Coatzacoalcos (Cz), which had elevated activity of a specific esterase designated CzEst9. The toxicological profile of the Cz strain had indicated that an esterase-mediated mechanism was involved in pyrethroid resistance instead of target site insensitivity. We have utilized a PCR assay to test for the pyrethroid resistance-associated amino acid substitution in the sodium channel gene of the Cz strain and a target site-mediated pyrethroid resistant strain, San Felipe (SF). Permethrin hydrolysis assays and esterase activity gels were utilized to assay for metabolic esterase-based resistance. The metabolic esterase CzEst9 was purified and the gene encoding CzEst9 cloned.

Materials and Methods

Rearing of ticks and bioassays were done at the USDA-ARS Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX as described by Davey *et al*. (1980). The sources, establishment and toxicological characterization of the strains were described by Miller *et al*. (1999). Bioassays were performed using the FAO standard larval packet test (FAO, 1984). Probit analysis, including probit transformation of percentage mortality and natural logarithm transformation of dose, was performed using the Polo-PC Program (LeOra Software, 1987). Resistance ratios were determined relative to bioassay data from the reference pyrethroid susceptible Gonzalez strain. To separate bioassay 'alive' and 'dead' larvae in the Cz and SF resistant strains, bioassays were conducted using five concentrations of permethrin, with each concentration repeated three times. When bioassays were read, 'alive' and 'dead' larvae at each concentration were separated with vacuum and immediately frozen at −70°C for molecular assays.

DNA was purified from individual tick larvae and PCR performed using a DNA Engine (MJ Research, Watertown, MA) as previously described (Guerrero *et al*., 2002). Reaction products were fractionated on 2.5% NuSieve agarose (BioWhittaker Molecular Applications, Rockland, ME) TBE gels and DNA visualized by staining with GelStar DNA Staining Dye (BioWhittaker Molecular Applications, Rockland, ME) and UV illumination. Esterase activity gels and quantitative analysis of esterase hydrolysis of α- and β-naphthyl acetate and permethrin were performed as reported by Jamroz *et al*. (2000).

Results

Table 1 summarizes larval bioassay and sodium channel PCR assay results for the *B. microplus* strains evaluated for this report. The two permethrin susceptible strains were almost completely homozygous for the wild type susceptible sodium channel allele, indicated by the high percentage of SS alleles in those populations. The sample from the highly resistant SF strain did not have any SS individuals and had a high level of homozygous mutated resistant (RR) individuals. Surprisingly, the resistant Cz strain was comprised almost entirely of individuals with the SS sodium channel genotype, indicating this strain must possess a resistance mechanism differing from that in the SF strain. To further investigate this possibility, esterase activity profiles of each strain were determined (Figure 1). The Cz strain possessed an intense esterase activity band (*), designated EST9 (Jamroz *et al.*, 2000), which was not found in the other three strains. This esterase activity pattern

Table 1. Permethrin challenge and bioassay data and PCR tests of various *B. microplus* strains[a]

Strain	Larval bioassay data			PCR[b]			
	Permethrin phenotype	LC_{50} (95% CL), %AI[d]	Res[c] ratio	No. tested	SS (%)	SR (%)	RR (%)
Gon	SUS	0.014 (0.013–0.015)	1	28	94	3	3
Tux	SUS	0.022 (0.020–0.026)	1.6	29	100	0	0
Cz	RES	3.45 (2.43–4.26)	250	26	92	8	0
SF	RES	25.8 (18.5–46.7)	1840	28	0	29	71

[a] From Guerrero *et al*. (2001).
[b] SS, SR and RR indicate homozygous susceptible, heterozygous, and homozygous resistant, respectively, for the sodium channel genotype.
[c] Res ratio factor, resistance factor = test LC_{50}/Gonzalez reference strain LC_{50}.
[d] Active ingredient permethrin.

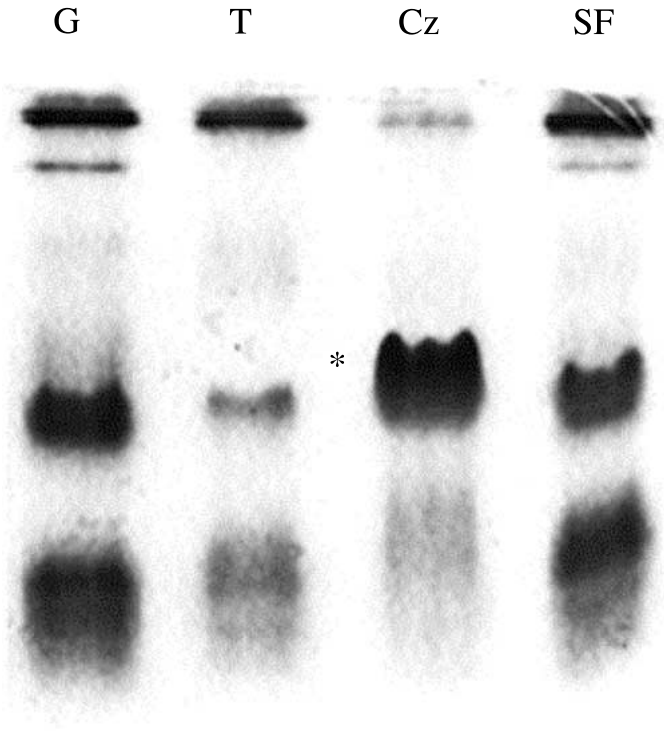

Figure 1. Native gel profiles of esterase activity in experimental *B. microplus* tick strains. Protein was extracted from 14-day-old, unfed larvae from the G, T, Cz, and SF strains and four larval equivalents per lane (6.5–8.0 μg protein) was fractionated on a 12% native polyacrylamide gel and stained in the presence of β-naphthyl acetate. (*) denotes CzEst9.

has consistently been replicated in assays of tick protein extracts from several generations of these strains and by varying the acrylamide concentration of the activity gel, a greater differentiation between various migrating esterase species can be achieved (data not shown). Other esterase activity gels run in the presence of eserine sulfate (Jamroz *et al.*, 2000), an inhibitor of acetylcholinesterase activity, and triphenylphosphate (data not shown), an inhibitor of carboxylesterase activity, have also shown that EST9 appears to be unique to the Cz strain.

CzEst9 protein was purified from Cz larvae, the purified fraction shown to have permethrin hydrolytic activity (Pruett *et al.*, 2002) and, following amino acid sequencing, found to have the nucleotide sequence reported by Hernandez *et al.* (2000) as clone 13. Hernandez *et al.* (2002) reported two alleles for clone 13 (CzEst9) which differed only by a single Asp → Asn amino acid substitution and a PCR-based assay to distinguish the alleles from each other and hypothesized this substitution played a role in the pyrethroid

Table 2. PCR analysis of sodium channel and CzEst9 mutations in SF and Cz strains of *B. microplus*

Strain	Dose[a] (%)	Bioassay mortality (%)	Sodium channel[b]						CzEst9[b]					
			Alive			Dead			Alive			Dead		
			SS (%)	SR (%)	RR (%)	SS (%)	SR (%)	RR (%)	SS (%)	SR (%)	RR (%)	SS (%)	SR (%)	RR (%)
SF	30.0	54	0	0	100	11	61	29	21	61	18	11	57	32
	3.75	20	7	46	46	71	25	4	14	54	32	11	50	39
	Untreated		5	27	68	–	–	–	24	38	38	–	–	–
Cz	15.0	95	0	0	100	32	57	11	0	12	88	4	26	70
	1.88	69	4	50	46	18	64	18	0	14	86	4	32	64
	Untreated		34	41	25	–	–	–	8	29	63	–	–	–

[a] Active ingredient permethrin.

[b] SS, SR and RR indicate homozygous susceptible, heterozygous, and homozygous mutant, respectively, for the sodium channel or CzEst9 genotype. Percentages of the 'alive' group are calculated using the number of a specific genotype (SS, SR or RR) divided by the total number of surviving larvae. Likewise for the 'dead' group.

resistance mechanism of the Cz strain. Later studies indicated a role of this amino acid substitution in this strain's pyrethroid resistance, though of lesser importance than the absolute level of EST9 (Guerrero *et al.*, 2002). To attempt to associate the wild type and mutated CzEst9 and/or sodium channel alleles with resistance to permethrin, bioassays were utilized to segregate the most resistant individual larvae from the most susceptible larvae within the SF and Cz strains. Table 2 shows the results of the sodium channel and CzEst9 diagnostic PCR assays on larvae from the SF and Cz strains, which survived or were killed by various doses of permethrin. In Table 2, the sodium channel and CzEst9 alleles containing the substituted amino acids hypothesized to be resistance-associated have been designated as 'R'.

Discussion

Experimental evidence indicates at least two different primary mechanisms of pyrethroid acaricide resistance in Mexican populations of *B. microplus*. The SF strain has a large number of individuals with sodium channel gene mutations, which suggest a target site insensitivity-mediated resistance. The Cz strain appears to predominantly utilize an esterase-mediated pyrethroid resistance mechanism. Earlier work using synergists (Miller *et al.* 1999) had suggested the carboxylesterase-mediated resistance in the Cz strain and this was supported by the presence of a highly active band of EST9 in the esterase activity gels (Figure 1). It is interesting that EST9 activity on native esterase activity gels has only been found in the Cz strain of ticks, despite testing 21 different *B. microplus* populations from various regions of Mexico (data not shown). It is evident from the PCR assays that in the SF strain the RR sodium channel genotype, or some factor which is associated with the RR genotype, is important to survival of permethrin challenge. At the 3.75% permethrin dosage, which killed only 20% of the total larvae, 71% of the dead larvae were SS for the sodium channel genotype. All of the survivors of the 30% permethrin dosage were RR for the sodium channel genotype. Regarding the SF strain's CzEst9 genotype, there was no clear trend, possibly because the target site insensitivity mechanism is so prevalent in the population. In the Cz strain, the survivors of the 15% permethrin dose, which killed 95% of the larvae were all RR for the sodium channel genotype. However at the lower dose of 1.88% permethrin, which killed 69% of the Cz larvae, the genotypes of the dead larvae were not predominantly SS type as occurred in the SF strain. This could be due to the overexpression of the CzEst9 enzyme in the Cz strain.

Hernandez *et al.* (2002) reported the CzEst9 transcript was 5-fold more abundant in the Cz strain compared to the G, T and SF strain. Jamroz *et al.*

(2000) used an HPLC method to determine the permethrin hydrolytic activity of the Cz strain. Compared to the susceptible reference G strain, Cz possessed over 5-fold greater permethrin hydrolytic activity. Thus, the Cz strain possesses more CzEst9 transcript, more esterase hydrolytic activity associated with the CzEst9 band on native esterase activity gels, and more permethrin hydrolytic activity than the susceptible G strain or the target site resistant SF strain. All of this supports a CzEst9 esterase-mediated permethrin resistance mechanism in the Cz strain. The CzEst9 mutated allele might play a role in resistance in the Cz strain, though the comparison between the CzEst9 genotypes of the alive and dead larvae (Table 2) do not show a strong selection in favor of the mutated allele in surviving larvae.

One final note of interest regards the differences in the sodium channel genotype of the Cz strain found in the experiments reported in Tables 1 and 2. The sodium channel PCR of generation F26 (Table 1) shows 92, 8, and 0% of the Cz larvae were SS, SR, and RR, respectively while the PCR of generation F36 (Table 2) shows 34, 41, and 25% were SS, SR and RR, respectively. This difference in sodium channel genotype distribution could be a result of Cz strain contamination in the interim between the generations with another *B. microplus* strain possessing the mutant sodium channel allele. It could also be the result of the permethrin pressure, which is applied at each generation of the Cz and SF strains, selecting for the mutant sodium channel allele. We do not presently have information which would allow us to distinguish between these two possibilities.

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